TREATMENT OF NON-NEURONAL CANCER
USING HSV MUTANT

FIELD OF INVENTION

5   The present invention relates to the treatment of
non-neuronal cancer tumors, particularly mesotheliomas,
melanoma, ovarian carcinoma or bladder cancer whether the
tumors are metastatic tumors or primary tumors.

BACKGROUND OF THE INVENTION

10   The DNA sequence of herpes simplex type 1 (HSV-1) is
known and is linear with a length of about 152k residues.
It consists of two covalently linked segments, designated
long (L) and short (S). Each segment contains a unique
sequence flanked by a pair of inverted terminal repeat

15   sequences. The long repeat (R_L) and short repeat (R_S)
are distinct. The unique long (U_L) region includes genes
UL1 to UL56, and the U_S region includes genes US1 to
US12.

20   The U_L region is flanked by a terminal repeat region
(TRL) and an internal repeat region (IRL) which lies
adjacent the IRS of the U_S region. Two genes RL1 and
RL2 lie within each of the repeat regions TRL and IRL.
The RL1 gene codes for the protein ICP 34.5, and this

25   gene is referred to herein as γ34.5.
       A number of naturally occurring and artificially-
engineered HSV-1 mutants have recently been identified
that appear to replicate preferentially in transformed
cells (Martuza *et al*, 1991; Mineta *et al*, 1994). Because

30   of the natural tropism of wild type herpes virus for
neuronal tissue, the published uses of modified,
replicating HSV to treat cancer have centered around
tumors of CNS (central nervous system) origin. Initial
experiments utilising HSV-1 mutants with deletions in the

35   thymidine kinase gene (HSV-TK⁻) showed dose dependent

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improvement in the survival of nude mice bearing human gliomas, medulloblastoma, malignant or atypical meningioma and neurofibrosarcoma both *in vitro* and in tumor bearing nude mice (Martuza *et al*, 1991; Markert *et al*, 1993). More recently, additional "replication restricted" non-neurovirulent mutants of HSV that contain the HSV-TK gene (a potential safety factor that would allow elimination of virus by treatment with the drug ganciclovir), but lack other HSV genes have been developed and have shown even more promise in CNS tumors.

A mutant HSV-1 called R3616, containing a 1000 base pair (bp) deletion in γ34.5, with LD_{50} (minimum dose of virus that kills 50% of infected animals) that is at least $3x10^3$ fold greater than wild type F strain virus from which it was derived, has been shown to improve the outcome of nude mice bearing intracranial human gliomas (Mineta *et al*, 1995).

In the work presented here, we have utilised an HSV-1 strain 17 mutant virus called 1716, that has a 759 bp deletion in each copy of γ34.5 of the long repeat region (R_L). The construction of mutant virus 1716 is described in published International patent application WO 92/13943 (PCT/GB92/00179) the contents of which are incorporated herein by reference. However, this patent publication is solely concerned with the use of mutant 1716 as a vaccine, either in itself or as a vector vaccine which includes a heterologous gene coding for an antigen.

WO 96/03997 (PCT/GB95/01791) includes data showing the efficacy of HSV 1716 against brain tumors.

SUMMARY OF THE INVENTION

The present invention is based on the surprising discovery that HSV which is γ34.5 null is effective against non-neuronal cancers. Since HSV is known to

selectively inhabit the neuronal system (including the peripheral and central nervous system) and where it may remain in a latent state, it was unexpected that an HSV mutant could be effective against cancers of non-neuronal origin. Moreover, replication of the HSV mutant *in vivo* is restricted to the tumor cells so that "normal" non-tumor cells are unaffected.

     Accordingly, the present invention provides the use of a mutant herpes simplex virus which has been modified in the $\gamma 34.5$ gene such that the gene is non-functional, in the manufacture of a medicament for use in treating a non-neuronal cancer.

     The invention also relates to a method of treating a non-neuronal cancer in a mammal, which method comprises the step of administering to the mammal an effective amount of a mutant herpes simplex virus which has been modified in the $\gamma 34.5$ gene such that the gene is non-functional. The invention further provides an agent for treating a non-neuronal cancer, comprising a mutant herpes simplex virus which has been modified in the $\gamma 34.5$ gene such that the gene is non-functional.

BRIEF DESCRIPTION OF THE FIGURES

     Figure 1 shows an HSV-1716 single step viral growth curve on human malignant mesothelioma cells. Innoculum at time 0 was 5,000 plaque forming units (PFU) of virus (multiplicity of infection, MOI=0.1). At twenty-four hours the amount of virus present had increased by four logs over the initial input innoculum.

     Figure 2 shows an MTT assay for human malignant mesothelioma cell viability as a function of time and varying MOI. The % of control growth is the ratio of mean MTT activity in infected cells (n=6 wells at each time point) to the activity in a matched uninfected cells (n=6 wells at each time point).

Figure 3 shows the mean tumor score in animals day 28 animals (tumor/HSV animals received 5 x 10^6 pfu HSV-1716 on day 14). The mean tumor score in the control group was 3.9 ± 0.1 versus a mean tumor score in the

5   treatment group of 1.4 ± 0.2 ($p<0.001$);

Figure 4 shows an HSV-1716 viral dose response survival study. SCID mice received 30 x 10^6 human malignant mesothelioma cells on day 0. Seven days later one animal was sacrificed to confirm tumor. The

10   remaining animals were randomized into four groups: control (n=11, culture media), low dose (n=10, 5 x 10^4 pfu HSV-1716), middle dose (n=10, 5 x10^5 pfu HSV-1716), and high dose (n=10, 5 x 10^6 pfu-1716);

Figure 5 shows virus titer following infection of

15   subconfluent monolayers of 1205 cells with 5,000 PFU of HSV-1716 or HSV17+; and

Figure 6 shows tumor volume of treated and control tumors at various times after viral therapy.

20   DETAILED DESCRIPTION OF THE INVENTION

For the purposes of the present invention "non-functional" means that the gene has been modified by deletion, insertion or substitution (or other change in the DNA sequence such as by rearrangement) such that it

25   does not express the normal product or a functionally equivalent product. The effect of the non-functionality of the gene is that the neurovirulence of the virus to the patient is substantially removed. Each of the two γ34.5 genes is non-functional.

30   Thus the invention relies on the finding that rendering the γ34.5 gene non-functional provides an HSV mutant which is particularly effective in destroying dividing non-neuronal tumor cells, whilst at the same time the HSV mutant does not replicate within normal non-

35   cancerous cells. It therefore has the potential to

provide a safe anti-cancer treatment.

Two types of herpes simplex virus are known HSV-1
and HSV-2 and either may be employed in the present
invention to provide the HSV mutant.   Inter-type
recombinants containing DNA from both types could also be
used. HSV-1 and HSV-2 mutants 1716, 1771, 2604, 2616 and
2621 are described herein.

The modification may be effected at any convenient
point within the γ34.5 gene, and such point generally
corresponds to a restriction enzyme site or sites.   The
modification may be within the BamHI s restriction
fragment of each R_L terminal repeat (corresponding to 0-
0.02 and 0.81 - 0.83 mu).   The modification is typically
a deletion of 0.1 to 3kb, particularly 0.5 to 2.5kb, and
especially 0.7 to 0.8 kb.   The simple insertion of a stop
codon is also effective in preventing production of the
ICP 34.5 protein.

The HSV genome also includes a number of other genes
which are non-essential to the successful culturing of
the virus. Their removal may further contribute to the
safety of the HSV mutant by further reducing
neurovirulence and reducing the likelihood of
recombination to the wild type.   It is, of course,
necessary to retain within the HSV mutant the ability to
culture the mutant so that the mutant is self-replicating
and stocks of the mutant can be grown in tissue culture.
Lethal modifications of the genome which remove the
ability to culture the HSV mutant are not acceptable,
unless the missing gene products can be provided to the
culture system in an alternative way e.g. by the use of a
complementing cell line containing a plasmid which
expresses the missing gene product.

Thus, in addition to the primary modification to the
γ34.5 gene of the R_L region, it may be advantageous to
also include in the HSV mutant one or more secondary

modifications which are generally within non-essential
genes unless the missing gene product can be provided in
an alternative way.

The present invention also encompasses the use of an
HSV mutant which includes in addition to the primary
modification, a secondary modification (for example
within Vmw65). The mutant may be derived from HSV-1 or
HSV-2.

In a similar way, other secondary modifications may
involve modification of the latency associated transcript
(LAT) promoter so as to render the promoter non-
functional and prevent transcription thereof.

Herpes simplex virus naturally infects the brain and
nervous system. It is therefore surprising that the HSV
mutant is effective against tumors outside the brain and
nervous system. Such tumors may be metastatic tumors
where the cancer cells originate elsewhere or may be
primary tumors. The non-neuronal cancer being treated
will generally be a primary cancer of non-neuronal cell
type, or a secondary cancer of non-neuronal cell type
which has metastasised to a non-neuronal (e.g. non-CNS)
location in the patient's body.

On the basis of the results presented herein, which
surprisingly show the ability of the HSV mutant to combat
non-neuronal tumors, it is believed that the anti-tumor
effectiveness of the HSV mutant extends to the treatment
of non-neuronal (e.g. non-CNS) cancers in general,
including the treatment of mesotheliomas, melanoma,
ovarian carcinoma, and bladder cancer. The condition of
a patient suffering from such a cancer can be improved.
The treatment is particularly applicable to primary
tumors which are localised, rather than metastatic
tumors. The efficacy of treatment according to the
invention employing the HSV mutant will depend on the
time after origination of the tumor at which the

treatment is initiated, but efficacy is improved by early
treatment for example in 1 to 30 days.

The LD_{50} (minimum dose of virus that kills 50% of
infected animals) of the 1716 mutant in respect of mice

5   is 10^6 fold greater than that of the wild type 17+ virus
from which it is derived (for cerebral tumors). Thus the
neurovirulence of 1716 is essentially removed relative to
the wild type virus.

The effective non-toxic dose of HSV mutant can be

10   determined by routine investigation by the skilled
addressee, and will depend on a number of factors
including the particular species of mammal and the extent
of development of the tumor. A guide can be obtained
from the Examples herein, which show that in the mouse

15   relatively high doses ($4x10^6$ pfu) can significantly
improve survival. Preferred doses for mice are in the
range $1x10^4$ to $1x10^8$ particularly $1x10^6$ to $1x10^7$ pfu. The
doses for other mammals can be estimated accordingly by
the skilled man. For humans doses will generally be in

20   the range $1x10^6$ to $1x10^8$ pfu.

In a further aspect of the invention there is
provided a method of treating non-neuronal cancer in
mammals, in particular in humans by administering a
pharmaceutical formulation comprising the HSV mutant to a

25   mammal, in particular to humans. Thus, the method of
treatment can comprise the administration of a
pharmaceutical formulation comprising the HSV mutant by
injection directly into the tumor, parenterally into the
blood stream feeding the tumor or intraperitoneally. The

30   tumor may be surgically removed or debulked prior to
treatment with the HSV mutant.

It will usually be presented as a pharmaceutical
formulation including a carrier or excipient, for example
an injectable carrier such as saline or apyrogenic water.

35   The formulation may be prepared by conventional means.

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The following Examples illustrate the invention.

EXAMPLE 1

MATERIALS AND METHODS

5   Mutants in the HSV RL1 gene

Our laboratory has isolated a number of deletion
mutants and point mutants in the RL1 gene of both HSV-1
strain 17 and HSV-2 strain HG52.

10   HSV-1 strain 17

1716

The genome of this virus has a 759 bp deletion
located within each copy of the BamHI fragment (0-0.02
and 0.81-0.83 map units) of the long repeat region of the
15   genome. The deletion removes one complete copy of the 18
bp DR1 element of the 'a' sequence and terminates 1105 bp
upstream of the 5' end of IE gene 1. Most of RL1
including the initiating methionine is removed and the
mutant fails to make ICP34.5. Following intracerebral
20   inoculation of mice, the LD_{50} value of 1716 is $7x10^6$/mouse
compared to <10 for the parental strain 17+.

Its production is described in published patent
application WO 92/13943 (PCT/GB92/00179). The mutant
virus was passaged for use in this study by Dr. Nigel N.
25   Fraser (Philadelphia, PA).

1771

The genome of this virus has a stop codon functional
only in the assigned RL1 reading frame 9 bp downstream
from the initiating ATG. The LD_{50} value of 1771 is $>10^6$
30   PFU/mouse following intracerebral inoculation and its
latency phenotype is indistinguishable from 1716. 1771
fails to synthesize ICP34.5.

HSV-2 strain HG52 mutants.

2604

35   This virus has a deletion of 1488 bp in both long

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repeats of the genome which extends from the DR1/Ub
boundary of the 'a' sequence to 511 bp upstream of the 5'
end of IE1. The deletion removes the whole of RL1. 2604
has a LD_{50} value of $>10^7$ PFU/mouse compared to $<10^2$ for
wild type strain HG52. Although formal proof of lack of
synthesis of ICP34.5 has not been obtained due to the
unavailability of an antiserum which detects the type 2
protein, the phenotype of the virus *in vivo* has been
shown to be unambiguously due to the RL1 deletion.

2616

This virus has 786 bp of both copies of RL1 deleted
but retains 782 bp upstream of the 5' end of IE1 and 463
bp downstream of the "a" sequence. The LD_{50} value of
2616 on intracerebral inoculation is $>10^6$ PFU/mouse.

2621

This virus has a stop codon inserted only in the RL1
open reading frame 9 bp downstream of the initiating
methionine within exon 1. The virus has a LD_{50} of $>10^7$
PFU/mouse following intracerebral inoculation.

In vitro studies of HSV-1716 on a Human Malignant
Mesothelioma Cell Line.

A human malignant mesothelioma cell line called REN
was isolated, characterised, and passaged as previously
described by our laboratory. To construct a single step
viral growth curve, REN cells were plated on six-well
plates at a density of 500,000 cells/well and infected 24
hours later with HSV-1716 at a multiplicity of infection
(MOI) of 0.01 (5000 pfu/well). One well was harvested at
0, 6, 12 and 24 hours by cell scraping and collection of
the media. The samples were freeze/thawed and titered on
Baby Hamster Kidney cell monolayers. A cell viability
assay was performed by plating REN cells in 96 well
plates at a density 20,000 cells per well. Twenty four
hours later the cells were infected with HSV-1716 at

MOI's of 0,0.001, 0.01, 0.1. Six wells were infected at
each MOI.   Three complete 96 well plates were
constructed to allow for viability assay at 24, 48 and 72
hours.   Viable cell number was assessed by a colorimetric
5   assay (Cell Titer 96% Aqueous Non-radioactive MTT Cell
Proliferation Assay; Promega Corporation, Madison, WI)
that measures viable cell dehydrogenase activity by
absorbance.   The present control growth is defined as the
ratio of the mean absorbance of six treatment wells at
10   490nm to the mean absorbance of six untreated matched
controls.

In vivo Studies
      A previously described model of human malignant
15   mesothelioma growing in the peritoneal cavity of SCID
mice was utilised for all _in vivo_ experimentation (Smythe
_et al_, 1994a; Smythe _et al_, 1994b).
      Briefly, SCID mice were obtained and housed at the
animal facilities of the Wistar Institute (Philadelphia,
20   PA).   On day 0, animals were injected intraperitoneally
with 30×10^6 REN cells in 1 cc of cell culture media.
For the tumor burden study treatment animals were given
4×10^6pfu of HSV-1716 in culture media by intraperitoneal
injection on day 14.   Control animals received an
25   equivalent volume of culture media.
      The animals were examined daily and sacrificed by
cervival dislocation on day 28.   The amount of tumor
burden was assessed using a four-point semiquantative
scale which accounts for both gross and microscopic
30   disease.   Briefly, animals were assessed for tumor in the
following four areas: stomach/pancreas, portal region,
retroperitoneum/diaphram, and small bowel mesentary.
      On gross examination animals received either a score
of 0 if no tumor was present or a score of 1 in each of
35   the four designated areas where gross tumor was seen.   If

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no gross tumor was visible, H & E stained paraffin
embedded sections of each organ from the designated area
were examined in a blinded fashion by a clinical
pathologist. The sections were scored as either 0 for no
5   microscopic tumor or 0.5 if microscopic tumor was
present. Thus, the tumor scores ranged for 0 to 4.0.
Organs including: brain, heart, lungs, liver, stomach,
pancreas, kidney, adrenals, spleen, gonads, small bowell,
and diaphram were obtained from each animal. Each organ
10   was divided by thirds with equal samples designated for
frozen section, formalin fixation and DNA extraction.

For the initial survival study, 18 animals were
injected intraperitoneally with 30×10^6 REN cells in 1
cc of cell culture media (day 0). On day 7, one animal
15   was sacrificed for gross tumor confirmation and the
remaining animals were randomized to the treatment group
(n=8) and the control group (n=9). Treatment animals
received 4×10^6 pfu of HSV-1716 by intraperitoneal
injection; control animals received an equal volume of
20   culture media. The animals were checked daily and
followed for survival. An identical protocol was
followed for the dose response study except the animals
were randomized into the control group (n=10), the high
dose group (n=10, 4×10^6 pfu HSV-1716), the middle dose
25   group (n=10, 4×10^5 pfu HSV-1716) and the low dose group
(n=10, 4×10^4 pfu HSV-1716).

Histology and Immunohistochemistry
Tissue samples were obtained at necropsy. A portion
30   of each specimen was fixed in 10% neutral buffered
formalin overnight, paraffin embedded, sectioned and
stained with hematoxylin and eosin for microscopic
examination. Immunohistochemical staining for HSV
infection was performed on frozen tissue sections with a
35   commercially available polyclonal antibody for cell

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surface HSV antigens (DAKO, Carpinteria, CA).

In vivo Dissemination and Restriction Studies

5   In order to look for dissemination of HSV-1716, we
performed PCR looking for the herpes virus thymidine
kinase gene (tk) on the collected tissues from two
animals in the tumor burden study. Genomic DNA was
obtained by standard phenol/chloroform extraction and
amplified by PCR. The PCR primers (5' ATGG CTTT TCGT
10   ACCC CTGC CAT AND 3' GGTA TCGC GCGG GGGG GTA) were
designed to span a region of the HSVtk gene generating a
536 bp fragment. Ten microliters of DNA extract from
each tissue sample was subjected to 35 cycles of PCR
using the tk primers. The tk plasmid as well as DNA
15   brain tissues from an animal infected with wild type HSV-
17+ were used as positive controls. PCR products were
run on ethidium bromide 1.5% agarose gels and then
blotted overnight onto Zeta-Probe GT Blotting Membranes
(Bio-Rad Laboratories, Hercules, CA). The membrane was
20   probed using a ^{32}P-labeled portion of the HSVtk plasmid
corresponding to the 536 bp PCR generated tk fragment.

RESULTS
HSV-1716 Efficiently Replicates in a Human Malignant
25   Mesothelioma Cell Line and Lyses the Cells *In Vitro.*

To determine the ability of HSV-1716 to replicate
within a non-CNS human tumor cell line in vitro, we
performed a single step viral growth curve in REN cells
(a human malignant mesothelioma cell line isolated and
30   characterised from a clinical specimen in our
laboratory). As shown in Figure 1, REN cells supported
rapid growth of the virus. At time 0, 70% of the input
viral innoculum was recovered. By 6 hours, the number of
recovered active viral particles fell by a factor of 200
35   as expected due to viral uptake and disassembly in

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preparation for viral replication. Twelve hours later,
viral recovery increased to a level near the input
innoculum. By 24 hours, a 4-log increase over the
initial innoculum was obtained demonstrating efficient
5   replication of HSV-1716 on REN cells.

To demonstrate the ability of HSV-1716 to lyse REN
cells, we next performed an in vitro target cell
viability assay. As shown in Figure 2, HSV-1716
efficiently lysed target cells in a time and dose-
10   responsive fashion. By 72 hours, at an MOI=1.0, less
than 20% of the cells remained viable when compared to
matched uninfected control cells. Similar results have
been obtained with a second human mesothelioma line, I-45
(data not shown).

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Unlike Wild-type HSV-1, HSV-1716 Infection and
Replication is Restricted to Tumor Cells in an *In vivo*
SCID Mouse Model of Human Mesothelioma.

As expected, intraperitoneal injection of SCID mice
20   with 5×10^6 pfu of wild type HSV-17+ led to rapid spread
of the virus, neurological dysfunction, and death of all
animals by 7 days. To determine the extent of HSV
infection, organs from animals sacrificed 72 hours after
wild type injection were analysed immunohistochemically
25   with a polyclonal antibody recognising HSV-antigens.
Positive cells were clearly seen in the myenteric ganglia
of the small intestine, adrenal glands, and brain. In
contrast, non-tumor bearing SCID mice injected with the
same dose of HSV-1716 remained healthy. Immunohisto-
30   chemistry for HSV antigens was negative 72 hours after
infection.

To test the ability of HSV-1716 to infect and
replicate within human tumors *in vivo*, SCID mice were
injected intraperitoneally with 30 million human REN
35   cells. After 14 days, diffuse macroscopic 5-8 mm tumor

nodules were present. At this time, 5x10^6 pfu of HSV-
1716 were instilled into the peritoneal cavity; 72 hours
later, the animals were sacrificed and the abdominal
organs processed for immunohistochemistry to detect HSV-
5   proteins. Microscopic examination revealed that
virtually all tumor nodules showed necrosis, infiltration
with mononuclear inflammatory cells, multinucleated cells
and nuclear inclusions consistent with active herpetic
infection. In contrast, no viral cytopathic changes were
10   seen in any normal tissues. To directly detect HSV
infection, tumors and organs were stained with an anti-
HSV antibody. A large percentage of the tumor cells
stained positively for HSV antigens while surrounding
normal tissues, as well as other normal tissues examined,
15   showed no positive staining. Specifically liver, kidney,
spleen, small bowel, myenteric plexuses, adrenal glands,
spinal cord and brain were negative. Similar results
were obtained at days 5, 7, 9 and 11 after infection,
however, the number of positive cells appeared to
20   decrease at the later time points, possibly due to a
decrease in available tumor substrate.

HSV-1716 Does Not Persist Following Intraperitoneal
Injection in Tumor-Bearing Mice.
25   To more sensitively detect the persistence and
dissemination of HSV-1716 after intraperitoneal
injection, we used the polymerase chain reaction (PCR) to
detect the presence of Herpes Simplex Thymidine Kinase
(HSVtk) DNA. The results from two tumor-bearing animals
30   demonstrated no HSVtk dissemination and no HSVtk
persistence two weeks after intraperitoneal injection.
In contrast, one animal who was given HSV-17+ as a
positive control, demonstrated HSVtk dissemination to the
brain within 72 hours after intraperitoneal injection.

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HSV-1716 reduces intraperitoneal tumor burden and
markedly prolongs survival in a SCID mouse model of human
mesothelioma.

To determine the ability of HSV-1716 infection to
erradicate established tumor, 5×10^6 plaque forming units
(pfu) of HSV-1716 were given by intraperitoneal injection
to animals that had been injected intraperitoneally 14
days previously with 30 million REN tumor cells. Animals
at this time had established intraperitoneal tumors that
consisted of multiple 5 to 8 mm nodules with portal
invasion and gallbladder distension. Two weeks later,
animals were sacrificed and the tumor burden was assessed
using a previously developed semi-quantitative scale
which accounts for both gross and microscopic tumor
(Hwang et al, 1995). The tumor score ranges from 0 (no
gross or microscopic tumor) to a maximum score of 4.0.
Figure 3 shows a significant reduction in the mean tumor
score at day 28 in tumor-bearing animals (n=12) treated
with HSV-1716 as compared to the mean tumor score in
control animals (n=8). The mean tumor score in the
treatment group was 1.4 ± 0.2 compared with a mean tumor
score in the control group of 3.9 ± 0.1 (p,0.001). All
animals in the control group survived the study period.
There was one death in the treatment group that occurred
5 days after viral administration. Gross examination
failed to reveal the cause of death; microscopic
examination was not possible due to autolysis.

To determine if this decrease in tumor mass
conferred a survival advantage to SCID mice bearing
established intraperitoneal REN tumors, a second set of
tumor-bearing animals were injected with 5×10^6 pfu of
HSV-1716 two weeks after intraperitoneal injection of
tumor cells and followed for survival. The median
survival was increased from 47 days in the control group
(n=9) to 95 days in the treatment group (n=10). After

102 days, the remaining 3 surviving animals were
sacrificed and necropsied. All deaths in the control
group were a result of bulky intraperitoneal disease; no
external tumor nodules were visible at the initial tumor
5   injection site. Interestingly, deaths in the treatment
group occurred at two distince time points. Three
animals died shortly after HSV-1716 administration.
There was no evidence of bulky disease at this time. The
majority of the other animals died around day 100 due to
10   bulky malignant disease that extended from large
subcutaneous nodules arising on the anterior abdominal
wall. These nodules corresponded to the site of the
initial tumor injection and the tumor appeared to be
growing inward from the anterior abdominal with invasion
15   into the peritoneal cavity. There were no deaths in a
cohort of non-tumor bearing animals (n=5) who received
the same dose of HSV-1716 by intraperitoneal injection.

A second survival study was performed to determine
the viral dose response. Tumor bearing animals were
20   randomised to control (n=11) and treatment groups (low
dose - 4×10^4 pfu HSV-1716, n=10; middle dose - 4×10^5 pfu
HSV-1716, n=10; and high dose - 4×10^6 pfu HSV-1716,
n=10). As shown in Figure 4, treatment with high dose
HSV-1716 significantly improved survival when compared to
25   control animals (p=0.0011 by Mantel-Cox logrank test).
Seventy percent of the high dose animals were alive at
day 90; however, 5 out of the 7 developed subcutaneous
tumor nodules on the anterior abdominal wall
corresponding to the initial tumor injection site. The
30   low and middle dose treatment animals also demonstrated a
significant improvement in survival when compared to the
control animals (p=0.0003 for control vs. middle dose and
p=0.0019 for control vs. low dose by Mantel-Cox logrank
test). There was no difference in survival between the
35   low and middle dose animals (p=0.65).

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DISCUSSION OF RESULTS

These results demonstrate that the mutant "replication-restricted" Herpes Simplex Virus-1716 can reduce tumor burden and significantly prolong survival in an animal model of non-CNS localised human malignancy. Furthermore, we have shown that the HSV-1716 mutant is "replication-restricted" to malignant cells, in that it does not disseminate or persist after intraperitoneal injection into SCID mice bearing human tumors. These findings suggest that this virus is efficacious and safe for use in localised human malignancies of non-neuronal origin such as malignant mesothelioma, ovarian carcinoma, or bladder cancer.

The "replication-restriction" of HSV-1716 is provided by deletion of the γ34.5 gene. The function of this gene is still unclear, however, the loss of the γ34.5 gene has been shown correlate with a loss of neurovirulence.[24] There are also likely additional functions of this gene that allow for the restricted replication seen in our malignant mesothelioma cell lines.

EXAMPLE 2

MATERIALS AND METHODS

Studies of HSV-1716 on a Human Melanoma Cell Line

The use of replication restricted HSV-1716 to treat experimental human malignant melanoma outside the CNS was investigated as follows. Tests were carried out on mice in vivo.

Tumor Cells:

Human melanoma cell lines were a generous gift from Meenhard Herlyn (Wistar Institute, Phila, PA). Line 1205 was isolated as previously reported (Juhasz et al., 1993) and the other 25 lines were isolated in a similar

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fashion. Cells were grown in plastic flasks in AUTO-POW
media containing penicillin, streptomycin, and 5% calf
serum at 37°C in a humidified environment with 5% CO_2.

5   Virus Production:

        To produce virus stocks, subconfluent monolayers of
baby hamster kidney 21 clone 13 (BHK) cells were infected
with HSV strains 1716 or wild type 17+. Virus was
concentrated from the culture and titrated by plaque
10   assay (Spivack & Fraser, 1987). All viral stocks are
stored frozen at -80°C, and thawed rapidly just prior to
use.

        Production of UV Inactivated Virus:

15   Ultraviolet inactivation of HSV was performed using
the method of Notarianni and Preston (Notarianni &
Preston, 1982). After inactivation, the viral suspension
was titered on BHK cells to confirm that it could no
longer establish a lytic infection, stored frozen at
20   -80°C, and thawed rapidly just prior to use.

        Viral Growth Kinetics Assay

        Subconfluent monolayers of 1205 cells (5×10^5 cells
in six well plastic tissue culture plates) were infected
25   with 5×10^3 PFU of HSV-1716 in 1 ml of AUTO-POW media
containing penicillin, and streptomycin. For the time 0
measurement the cell monolayer was scrapped off into the
viral inoculum suspension immediately, and frozen at -
80°C. For the remaining time points, the viral inoculum
30   was incubated on the cell monolayer at 37°C for one hour
with gentle rocking, and then aspirated off. The
infected monolayers were washed twice with media, and
resuspended in 1 ml of AUTO-POW media containing
penicillin, streptomycin, and 5% calf serum. At the
35   appropriate times post infection, the monolayers were

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harvested with a cell scraper, and the suspension frozen at -80°C. Following 2 cycles of freezing and thawing, each sample was titrated by plaque assay on BHK cells (Spivack & Fraser, 1987).

Intracutaneous Tumor Production and Assays:

Mice were anesthetized with intramuscular ketamine/xylazine (87mg/kg ketamine/13mg/kg xylazine). A patch of hair was removed from one flank using a chemical depilatory agent (Magic Shaving Powder, Carson Products Co., Savannah, GA) Intracutaneous injection of 1×10^5 1205 melanoma cells in a total volume of 50µL was performed using a Hamilton syringe, and a disposable 28 g needle. Tumor volumes were calculated based on a radius obtained from orthogonal measurements of tumor diameters using a micrometer-caliper, and assume a spherical tumor shape. On day 14 post tumor cell injection mice were randomly divided into three groups. One group was treated with a 25µl intratumoral injection of 2.5×10^6 PFU of HSV-1716 using a Hamilton syringe, and a disposable 30g needle. The second and third groups were injected respectively with an equal volume of UV-inactivated HSV-1716 or viral culture medium alone as comparisons.

Immunohistochemistry:

Viral antigen expressing cells were detected by the indirect avidin-biotin immunoperoxidase method (Vector Labs, Burlingam, CA) as specified by the manufacturer with slight modifications developed in our laboratory (Gesser et al., 1994). Rabbit antiserum to HSV-1 (Dako Corp., Carpinteria, CA) was used at a dilution of 1:1000.

Statistical Analysis:

Data analysis including calculations of means, standard deviations and ANOVA was performed using

StatView statistical software (Abacus Concepts, Berkeley
CA) on an Apple MacIntosh computer (Cupertino, CA).

RESULTS

HSV-1716 and HSV-17+ replicate efficiently in human
melanoma cells.

Subconfluent monolayers of 1205 cells were infected
with 5×10^3 PFU of HSV-1716 or HSV17+. For the time O
measurement the cell monolayer was scrapped off into the
viral inoculum suspension immediately, and frozen. For
the remaining time points, the viral inoculum was
incubated on the cell monolayer at 37°C for one hour with
gentle rocking, and then aspirated off. The infected
monolayers were washed twice with media, and resuspended
in 1 ml of AUTO-POW media containing penicillin,
streptomycin, and 5% calf serum. At the appropriate
times post infection the monolayers were harvested with a
cell scraper, and the suspension frozen at -80°C.
Following 2 cycles of freezing and thawing, each sample
was titrated by plaque assay on BHK cells. The results
are shown in Figure 5. The data shown represent the mean
± standard deviation of triplicate determinations at each
time point.

Replication of HSV-1716 is restricted to Melanoma Cells
following injection into pre-formed intracutaneous tumor
nodules (data not shown).

Intracutaneous tumors were produced by injection of
1×10^5 1205 melanoma cells into SCID mice. On day 14 post
tumor cell injection, some mice were treated with an
intratumoral injection of 2.5×10^6 PFU of HSV-1716,
while control mice received an equivalent volume of UV
inactivated virus. Sections were taken from a control
tumor treated with UV inactivated 1716, and harvested 10
days later. Sections were also taken from a tumor

treated with HSV-1716, and harvested 5 days later; and
harvested 10 days later. The tumor and surrounding skin
were excised, fixed, stained immunohistochemically for
HSV-1, and counter stained with hematoxylin.

Intratumoral Treatment of pre-formed intracutaneous
melanoma with HSV-1716 causes a significant inhibition of
tumor progression.

        Anesthetised mice were injected intracutaneously
with 10^5 melanoma cells. On day 15 post tumor cell
injection, mice were randomly divided into three groups.
One group was treated with a 25μl intratumoral injection
of 2×10^6 PFU of HSV-1716, and animals in the two control
groups were injected with either an equal volume of UV-
inactivated 1716, or viral culture medium. The results
are shown in Figure 6. The data shown represent the mean
± standard deviation of 10 mice at each point.

DISCUSSION OF RESULTS

        As an initial step in assessing the viability of HSV
based therapy of human intracutaneous melanoma, the
efficiency of HSV-1716 replication was determined for
human melanoma cell line 1205, and compared to the
replication of HSV-17+, the parental strain from which
HSV-1716 was derived. As shown in Figure 5, the
replication cycle of HSV-1716 in these cells is
approximately 24 hrs, and each replication cycle yields
an approximately 4 log fold increase in lytically active
virus. The kinetics of virus production were nearly
identical for HSV-1716 and HSV-17+. An additional 25
human melanoma cell lines were tested for lytic
replication of HSV, and all of these replicated HSV-1716
and HSV-17+ efficiently in vitro (data not shown).

        We next evaluated whether replication of HSV-1716
could be demonstrated *in vivo* in pre-formed

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intracutaneous human melanoma nodules growing in SCID
mouse skin, and importantly we evaluated if replication
of the virus was detectable in surrounding normal
tissues. This was accomplished by immunohistochemical
5   evaluation of treated tumors and surrounding tissues.
Immunohistochemical staining of intracutaneous 1205
melanoma nodules with antibody to HSV at 5 days or 10
days after intratumoral injection of HSV-1716
demonstrated positive staining that was dispersed
10   throughout a large area of the tumor. At these times a
significant portion of the HSV-1716 treated tumor nodules
were necrotic. Moreover, no staining was seen in normal
murine tissues surrounding the tumor nodules in these and
all other sections examined. No staining, and no
15   significant necrosis was seen in the representative
control tumor treated with UV-inactivated HSV-1716 and
subjected to the full immunohistochemical protocol.

        After determining that HSV-1716 exhibits restricted
replication within intracutaneous melanoma, we performed
20   intratumoral therapy on pre-formed tumors, to test the
efficacy of this approach. Figure 6 shows the tumor
volume of treated and control tumors at various times
after viral therapy, and demonstrates that HSV-1716
significantly inhibits progression of pre-formed
25   intracutaneous melanoma. UV inactivated HSV-1716 had no
effect on tumor progression relative to nodules injected
with viral culture medium alone. Of the ten tumor
bearing mice treated with HSV-1716, three had complete
involution of melanoma nodules such that no palpable
30   tumors were present from day 21 on. ANOVA analysis of
the tumor volume data demonstrated that the effect of
HSV-1716 treatment is statistically significant
(p<0.0001) at all times post treatment examined. In none
of the HSV-1716 treated animals was there any mortality
35   or morbidity noted.

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It is therefore apparent from Figure 6 that HSV-1716 has the ability to reduce melanoma tumor size *in vivo* in mice into which melanoma tumors have been introduced by intracutaneous injection.

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